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Review

The function and characteristics of tyrosyl radical cofactors

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Abstract

Amino-acid radicals are involved in the catalytic cycles of a number of enzymes. The main focus of this mini-review is to discuss the function and properties of tyrosyl radical cofactors. We start by briefly summarizing the experimental studies that led to the detection and identification of the two redox-active tyrosines, denoted Y_Z and Y_D, found in the water-oxidizing photosystem II (PSII) enzyme. More recent work that shows that the histidine-cross-linked tyrosine located in the active site of cytochrome *c* oxidase forms a radical during the catalytic oxygen–oxygen bond-cleavage process is also described.

Advanced spectroscopic and structural studies have been performed to investigate the spin-density distribution, the protonation state and the hydrogen bonding of redox-active tyrosines. These studies have shown that the radical spin-density distribution is highly insensitive to the environment and that it is typical of a deprotonated species. In contrast, the hydrogen bonding and the nature of the proton acceptor or network of acceptors vary substantially in different systems. This is important for the function of the tyrosyl radical, as will be emphasized in a detailed discussion on the proposed function of Y_Z as a proton coupled electron-transfer cofactor in photosynthetic water oxidation.

Amino-acid radical enzymes are typically large complexes containing multiple subunits, chromophores and redox cofactors. The structural and mechanistic complexity of these systems has hampered the detailed characterization of their radical cofactors. In the final section of this mini-review, we will describe a project aimed at investigating how the protein controls the thermodynamic and kinetic redox properties of aromatic residues by using de novo protein design. Two model proteins of different size have been constructed. The smaller protein is a 67-residue three-helix bundle containing either a single buried tryptophan or tyrosine residue. The high-resolution NMR structure of the tryptophan-containing protein, denoted α_3W, shows that the aromatic side chain is involved in a π-cation interaction with a nearby lysine. The effects of this interaction on the tryptophan reduction potential were investigated by electrochemical and quantum mechanical methods. The calculations predict that the π-cation interaction increases the potential, which is consistent with the electrochemical characterization of α_3W. A larger 117-residue four-helix bundle, α_4W, has more recently been constructed to complement the work on the three-helix-bundles and expand the family of model radical proteins.

Keywords: Amino-acid radical; Tyrosyl radical; Oxygen evolution; Water oxidation; De novo protein; Protein design

1. Introduction

The research of Gerald T. Babcock and coworkers was focused on understanding the O_2/H_2O redox chemistry in nature. This work led to the unexpected discovery that tyrosyl radicals are involved in this biochemistry and it linked photosystem II (PSII) and cytochrome *c* oxidase to the recently recognized family of amino-acid radical enzymes. The key experiments that led to the detection and identification of the redox-active tyrosines in PSII and cytochrome *c* oxidase are briefly summarized in the first section of this mini-review. A common pattern with respect to the protonation state and the spin-density distribution has emerged from detailed spectroscopic studies of tyrosyl radicals. In contrast, the hydrogen-bonding milieu varies between different systems. These characteristics of tyrosine redox cofactors will be discussed. Tyrosine oxidation and reduction are strongly coupled to protonic reactions at the phenol head group in both solution and in proteins. This realization, along with other observations, form the basis for mechanistic models in which Y_Z in PSII and the cross-linked histidine-tyrosine moiety located at the active site of

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cytochrome *c* oxidase are functionally involved in proton-coupled electron transfer. Here we focus on the function of Y_Z and the proposal that this residue is essential for both electron and proton transfers during the catalytic cycle of PSII. In the final section of this article, we describe the construction of de novo proteins designed to investigate how specific structural features of the protein matrix influence the redox properties of aromatic amino-acid radicals.

2. Tyrosyl radicals in PSII and cytochrome *c* oxidase

PSII uses light energy to drive water oxidation in oxygenic photosynthesis [1,2]. In this process electrons are extracted from H_2O for CO_2 reduction, protons are released into the thylakoid lumen forming a chemiosmotic gradient, and O_2 is formed as a byproduct. In the early seventies, Babcock and Sauer investigated light-induced reactions in dark-adapted chloroplasts by time-resolved EPR spectroscopy. Based on this work, they proposed that the well-known EPR Signal II represents a dark-stable radical, later denoted D, formed by slow electron transfer to either the S_2 or the S_3 redox state of the manganese cluster [3]. In a following study, thylakoid membranes were deprived of their O_2-evolving activity by a variety of different treatments including elevation of the pH, the temperature or the salt concentration. A light-induced, reversible phase of Signal II was observed in all inhibited samples [4] and this kinetic component was assigned to cofactor Z, the physiological electron donor to P_{680}^+ [5]. A Signal II transient assigned to Z was subsequently also detected in O_2-evolving samples [6,7].

The work that followed demonstrated that Z was the single redox cofactor between P_{680}^+ and the substrate-binding manganese complex and disclosed the chemical identity of the D and Z species. Thus, in 1976 Babcock et al. [8] showed that the reduction rate of $Z^{\bullet}$ varied with the redox state of the manganese cluster, and that in the final $S_3 \rightarrow S_0$ reaction $Z^{\bullet}$ reduction was rate-limiting for O_2 formation. A few years later, Berthold et al. [9] developed a method to prepare PSII-enriched membranes, and this type of sample was used to investigate how Z oxidation correlates with P_{680}^+ reduction as a function of pH. An EPR investigation of inactivated PSII samples showed that the appearance of $Z^{\bullet}$ and the decay of P_{680}^+ have the same pH dependence [10]. This result was confirmed in O_2-evolving samples using optical spectroscopy [11]. It was concluded that the oxidizing equivalents generated at P_{680} were transported to the manganese complex via Z. With respect to cofactor arrangement, the conclusions derived from these early spectroscopic studies are consistent with recent structural work [12,13]. That $Z^{\bullet}$ and $D^{\bullet}$ are tyrosyl radicals was revealed by preparing samples from cyanobacteria cultivated on media containing deuterated tyrosine and monitoring the reduction in the hyperfine structures of their EPR spectra. Using site-directed mutagenesis, Z and D were subsequently identified as tyrosine 161 of the D1 and D2 reaction-center proteins, respectively (see Ref. [14] and references therein), and they are now frequently referred to as Y_Z and Y_D.

In aerobic respiration, O_2 is reduced to H_2O at the copper/heme-containing cytochrome *c* oxidase enzyme, which couples the free energy released during the redox process to proton translocation across the inner mitochondrial membrane [15,16]. Since the late seventies, Babcock and co-workers [15] worked to develop time-resolved Raman techniques to study redox proteins and, specifically, to identify and characterize intermediates in the catalytic cycle of cytochrome *c* oxidase. In 1998, Proshlyakov et al. [17] showed that substrate O_2 is cleaved in a single step and proposed that this event generates ferryl heme a_3, cupric Cu_B and a protein radical. In a subsequent study, iodide labeling and protein sequencing techniques were used to show that a protein radical indeed is formed during the bond-cleavage step and localized the radical species to the histidine-cross-linked tyrosine located at the active site [18]. These results, combined with data from other groups, provided a great stimulus for recognizing the active-site histidine-tyrosine unit of cytochrome *c* oxidase as a catalytically essential redox cofactor [16–21].

3. Characteristics of tyrosyl radical cofactors

In addition to PSII and cytochrome *c* oxidase, tyrosyl radicals have been detected in a number of enzymes including the class I ribonucleotide reductases (RNR), prostaglandin H synthase, catalase and photolyase [22]. In addition, a cysteine-cross-linked tyrosyl radical is present in the copper-containing active sites of galactose oxidase and glyoxal oxidase [23]. Multiple electron magnetic resonance spectroscopic techniques, operating in both continuous-wave and pulsed mode and at various frequencies, have been used to investigate the spin-density distribution, the protonation state, and combined with FTIR spectroscopy and structural studies, the hydrogen bonding of the tyrosyl radicals in these enzymes [22,23]. By preparing samples containing tyrosine selectively labeled with ^2H, ^{13}C or ^{17}O isotopes, the spin-density distribution has been determined for $Y_D^{\bullet}$ [24,25], $Y_Z^{\bullet}$ [26], $Y^{\bullet}(122)$ in *Escherichia coli* RNR [27] and $Y^{\bullet}$ generated in frozen alkaline solution by UV-light irradiation [28]. The same basic odd-alternate pattern is found in all system studied thus far. The distribution of the unpaired spin is essentially invariant between the different tyrosyl radicals and it is typical of a deprotonated species. This is not surprising since phenolic compounds like tyrosine become strong acids upon oxidation ($pK_A^{red} \sim 10$, $pK_A^{ox} < 0$). Moreover, the reduction potential of the tyrosyl cation $Y^{\bullet +}/Y$ redox pair is estimated to 1.38 V in water [29] and it is likely to be even higher in a low dielectric protein environment [30]. Thus, the tyrosine redox couples that are biologically relevant are $Y^{\bullet}/Y$ and $Y^{\bullet}/Y^-$. For most of the tyrosyl radical enzymes the amino-acid redox cofactor is

protonated in its reduced form and, consequently, the redox chemistry occurring at the tyrosine sites involves proton-coupled electron transfer.

The hydrogen bonding and the nature of the proton acceptor or network of acceptors vary among the different enzymes [22]. Indeed, the hydrogen-bonding pattern differs substantially even within the class I RNR enzyme family. These enzymes are composed of two homodimeric subunits denoted R1 and R2. The former contains the active site while the radical-generating tyrosine/di-iron center is located on the latter. In RNR from mouse and herpes simplex virus the radical is hydrogen bonded while, in contrast, in RNR purified from *Salmonella typhimurium* or *E. coli* it is not (see Ref. [31] and references therein). Interestingly, in the *E. coli* enzyme the redox-active tyrosine is hydrogen bonded to the metal site in its reduced state but this interaction breaks upon radical formation [31]. Structural studies on the *E. coli* R2 subunit reveal that the tyrosyl oxygen is located at a distance about 3.2 Å from one of the carboxyl oxygens of Asp-84. The asparate is in turn ligated to one of the irons in the binuclear metal site. Following radical formation via oxygen activation at the di-iron site, the aromatic side chain shifts and the distance between the tyrosyl oxygen and aspartate oxygen increases to >4 Å. As noted by Högbom et al. [31], the disconnection of the tyrosyl radical from the metal site might be a contributing factor to the remarkable stability of the radical. The observed structural change may be an example of kinetic control of side-chain radical chemistry in proteins: Oxidation coupled to deprotonation is facilitated by the hydrogen-bonding interaction to the metal site, whereas the hindrance to reprotonation slows the reduction of the radical.

A second observation recently made by Nordlund and colleagues is that there appears to be variability in the distance between the redox-active tyrosine and the metal site in RNR [32]. The class I enzymes are divided into two subgroups denoted Ia and Ib. The *E. coli* enzyme belongs to the former class and in this system the distance between the oxygen of the reduced tyrosine and the closest metal ion is about 5.3 Å. In the Ib enzyme from *Corynebacterium ammoniagenes* the phenol oxygen/metal distance is increased to about 7 Å. Nevertheless, the insertion of a water molecule between the tyrosine oxygen and the oxygen of the aspartate iron ligand maintains a hydrogen-bonded chain connecting the phenolic oxygen of the reduced tyrosine to the metal site. A bridging water molecule is also observed in the structure of the fully reduced R2 subunit from *S. typhimurium*, which also belongs to the Ib class. As suggested by the authors, the longer tyrosine/metal distance and the bridging water molecule may be a general feature of the class Ib enzymes [32].

That Y_D· is a hydrogen-bonded radical with low solvent accessibility was shown early on by the Babcock group [14]. The identification of the hydrogen-bonding partner as D2-H190 was first suggested by EPR studies on site-directed PSII mutants [33,34] and later proven in a pulsed electron nuclear double-resonance experiment using ^{15}N-labeled samples [35]. FTIR studies have shown that Y_D is hydrogen bonded also in its reduced state [36] and it appears likely that D2-H190 is the donor. As discussed in more detail below, Y_Z is situated next to the catalytic $(Mn)_4/Ca$ complex and its local environment is strongly affected by the presence and intactness of the metal site. Multiple exchangeable protons are found close to Y_Z· in both apo-PSII and in samples specifically depleted of calcium [37]. Studies from several laboratories have shown that in apo-PSII the radical site is highly disordered and accessible to solvent (see Ref. [38] and references therein). The Y_Z· site is more ordered and shielded from the bulk solution in calcium-depleted PSII in which the manganese cluster is retained [37]. The hydrogen-bonding status of oxidized or reduced Y_Z in the fully active enzyme is not well characterized, although kinetic studies have shown that D1-H190 has a strong influence over the redox properties of Y_Z [39,40].

4. Y_Z as an electron/proton transfer cofactor

As indicated above, kinetic spectroscopic evidence favored Y_Z as the single redox-active cofactor between P_{680} and the manganese complex. Thus, the function of Y_Z was believed to be to transfer electrons from the manganese cluster to P_{680}^+ [14]. Evidence began to accumulate, however, that suggested its involvement in water oxidation might be more intimate.

Illumination of PSII samples that have been inhibited by treatments such as calcium-depletion blocks the catalytic cycle in a S_2Y_Z· state. This state gives rise to a broad EPR signal that is attributed to the tyrosyl radical interacting magnetically with the manganese complex [41]. The degree of broadening indicates a distance between the two paramagnetic centers of less than 10 Å. The close proximity of Y_Z and the manganese complex is a feature of recent models of the electron density of PSII obtained from X-ray diffraction [12,13], and it opens the possibility that Y_Z participates intimately in the water oxidation chemistry.

A likely consequence of oxidation of Y_Z or Y_D is the loss of the phenolic proton to the nearby basic residues. As described above, the symmetrically disposed histidines D1-H190 and D2-H190 have received the most attention as possible proton acceptors, although other amino acid residues are certainly involved in determining the overall properties of the active site. Current models of the PSII electron density are not in agreement about the separation between Y_Z and D1-H190 [12,13]. The pK_As of the proton acceptor and donor molecules that are interacting with Y_Z and Y_Z· are likely to be one factor that determines the reducing strength of the tyrosine, and the oxidizing strength of the tyrosine radical (see, for example, Ref. [42] for the effect of pH on Y_Z in the S_3 state). Numerous recent investigations in apo-PSII show that the kinetic properties

of Y_Z are determined by the action of the basic residues that can accept the phenolic proton upon oxidation or resupply it upon reduction (for reviews, see Refs. [38,40]).

An intriguing question is the actual fate of the phenolic proton upon oxidation of Y_Z. Experiments using high concentrations of amphiphilic pH-indicator dyes showed that proton release from the lumenal side of PSII occurs prior to $Y_Z\cdot$ reduction. The interpretation of this observation is controversial. One explanation invokes the deprotonation of Y_Z and subsequent proton transfer through a hydrogen-bonded network connecting Y_Z with the aqueous phase [30,38]. In this model, the domino deprotonation event conveys the protonic charge from the Y_Z site to the lumen. An alternate explanation suggests that protons are released from the protein surface due to electrostatic repulsions, first with P_{680}^+ and later with a protonated, positively charged base formed and trapped during Y_Z oxidation [43,44]. In this latter case, the phenolic proton of Y_Z rebinds to the tyrosine upon its reduction.

Observations of the microsecond phases of P_{680}^+ reduction favor the former interpretation [30,38]. The faster phases of P_{680}^+ reduction by Y_Z occur in less than 1 μs in all four S-states. The slower 5–100-μs phases are particularly pronounced in the S_2 and S_3 states. These reflect the shifting of the equilibrium between Y_Z/P_{680}^+ and $Y_Z\cdot/P_{680}$, which is most readily explained by the progressive increase in the effective pH near $Y_Z\cdot$ as the positive charge of the proton is conducted towards the aqueous phase. Solvent deuterium kinetic isotope effects on the microsecond reaction are consistent with this interpretation. Thus, under physiological conditions and on the slower time scale in which oxidation of the manganese complex occurs, $Y_Z\cdot$ is a neutral radical in a site whose pH is approaching that of the bulk aqueous phase.

A typical reaction of phenoxyl and other organic radicals is hydrogen-atom abstraction [45]. This fact, connected with other observations, led to the hypothesis that $Y_Z\cdot$ functions by abstracting both electrons and protons from the substrate/manganese complex [1,46]. The coupled transfer of an electron and a proton from the manganese complex to $Y_Z\cdot$ will form an O–H bond worth 87 kcal/mol (365 kJ/mol) at the cost of one O–H bond of the substrate/manganese complex. Model manganese complexes and comparable theoretical studies indicate that the O–H bond enthalpy of an aquo or hydroxo ligand to manganese is in the range of 77–87 kcal/mol (320–365 kJ/mol) (for a summary, see Ref. [46]). Entropy changes for hydrogen-atom transfers are near zero, so e^-/H^+ transfers from the substrate/manganese complex to $Y_Z\cdot$ are expected to be weakly exothermic and spontaneous ($\Delta G<0$) [1,46]. Analysis of the kinetic properties of hydrogen-atom transfers between oxygen atoms, as would occur between the substrate/manganese complex and $Y_Z\cdot$, establishes the kinetic competence of these reactions [1] to add to their demonstrated thermodynamic competence.

The location of Y_Z relative to the manganese cluster has therefore been of great interest. The models of the electron density determined by X-ray diffraction have not yet achieved a high resolution, and many features are lacking, but locations of the manganese complex, Y_Z and Y_D have been assigned [12,13], though these assignments should probably be considered tentative. The tyrosines are at the edge of the transmembrane region, as expected, with the rings oriented so their phenolic oxygens point away from the membrane and toward the aqueous phase. The phenolic oxygen of Y_Z points toward the manganese complex. The metal structure is indistinct and its nearest manganese atom lies perhaps as close as 6.5 Å from the phenolic oxygen [13].

This distance, should it ultimately prove to be correct, appears to argue against the most direct form of coupled electron/proton transfer from a manganese ligand to $Y_Z\cdot$. A bridging OH group, perhaps from a water molecule, would readily enable the reaction, however, by both donating and accepting a proton. As noted above, the recently crystallized R2 subunit from *C. ammoniagenes* RNR has 7 Å and an extra water molecule between the tyrosine and the di-iron site compared with the 5.3 Å in the *E. coli* enzyme, yet both oxidize the tyrosine by oxygen activation and mobilize the radical for catalysis [32]. In addition, theoretical work suggests that a bridging water need not impede hydrogen-atom transfer [47].

The longer manganese–Y_Z separation is not truly a mechanistic disadvantage. Where tyrosyl radicals are formed adjacent to oxidized metal centers, a possibility exists that the radical may be covalently trapped by reaction with one of the metal ligands. The cross-linked tyrosine residues in cytochrome *c* oxidase and galactose oxidase are probably formed in this manner, and oxygen–oxygen bond formation in PSII may itself be a radical trapping reaction [48]. If $Y_Z\cdot$ were to add to a hydroxo or oxo ligand of the manganese complex, its phenolic ring would likely be hydroxylated as occurs in certain RNR mutants [49]. The separation between $Y_Z\cdot$ and the manganese complex may be necessary to prevent the inhibition of the reaction center that would occur should Y_Z be converted to dihydroxyphenylalanine.

The function of Y_Z as an abstractor of electrons and substrate protons from the substrate/metal cluster on all S-state transitions is consistent with the proton-release pattern of some PSII preparations in which one proton per transition is released. This simple mechanism allows the manganese cluster to have a constant electrical charge in all steps of the catalytic cycle. Other PSII preparations, however, exhibit S-state and pH-dependent patterns of proton release. Interpretation of these patterns remains controversial. Although uncompensated charge formed within the manganese complex has been proposed to explain these phenomena [44], structural changes occurring in the S-state cycle may cause Bohr protons to be taken up or released at various stages of the cycle [38]. These may be connected with binding of chloride ion or water molecules, since the affinity of the manganese complex for either of these depends upon S-state. As detected by mass spectrometry, at least one

substrate molecule is already bound by S_0 and two are observed by S_2 [50].

Another intriguing observation also suggests that Y_Z is involved intimately in water oxidation; the kinetics of $Y_Z\cdot$ reduction by S_3 matches the kinetics of O_2 release from PSII [8,51,52]. It has been suggested [46] that the equivalence of these rates indicates that the rate-determining step for the $S_3 \rightarrow S_0$ transition involves both hydrogen-atom transfer to $Y_Z\cdot$ and formation of the oxygen–oxygen bond on the manganese complex. This concerted mechanism easily accounts for the kinetic behavior, and it avoids the necessity of invoking additional chemical intermediates. Quantum chemical calculations on models for this reaction are in progress to test the feasibility of the proposed mechanism as the basis for dioxygen formation [53].

To summarize, the bulk of the evidence from many laboratories is consistent with the proposed role of Y_Z as a proton coupled electron-transfer cofactor in the water-oxidizing process catalyzed by PSII. The catalytic mechanism, and the extent by which Y_Z participate in the transfer of substrate protons from the active site to the thylakoid lumen, remains controversial however and will require further experimental and theoretical study.

5. De novo designed radical proteins

A common feature of amino-acid radical cofactors, which include tryptophan, tyrosine, glycine and cysteine residues, is that they are experimentally difficult to characterize. The sheer size and complexity of many amino-acid radical enzymes, combined with the often highly oxidizing nature of their radical cofactors, hamper electrochemical measurements. Other cofactors, noncatalytically active amino acids, or even the solvent, may be oxidized before the residue of interest. In addition, amino-acid redox cofactors have poor optical extinction coefficients and their spectroscopic features are easily hidden in spectra from proteins containing chromophores such as chlorophyll or heme. One method to circumvent these potential problems is by designing small, simplified model proteins in which, at the outset, multiple cofactors that may obscure the spectral or electrochemical properties of the amino-acid cofactor are avoided. Additional cofactors may be incorporated, in a stepwise manner, once the characteristics of the initial design have been determined.

Two model proteins of different size have been constructed with the aim to investigate the redox properties of tryptophan and tyrosine in a controlled and well-characterized protein milieu. The basic design of these radical protein scaffolds holds the following key features [29]: (i) The protein should be single-stranded and contain an unique Trp or Tyr residue. (ii) The aromatic side chain should be buried in the hydrophobic core of the protein. (iii) The remaining residues should be redox inert in order to isolate the radical chemistry to a single site. The smaller of the two radical protein scaffolds is a 65-residue three-helix bundle containing either a single tryptophan or tyrosine. The aromatic residue is placed in position 32, which, based on the design of the protein, is predicted to be located in the hydrophobic core. The polypeptide chains of the Trp- and Tyr-containing three-helix bundle, denoted α_3W and α_3Y, respectively, were first generated chemically by solid-phase peptide synthesis. An initial structural characterization provided data consistent with two stable, α-helical structures each containing a single aromatic side chain residing in a hydrophobic environment. In addition, the chemical-shift dispersion and narrow spectral line widths of their NMR spectra were consistent with uniquely structured proteins [29]. In order to derive the structure of the three-helix bundle scaffold by NMR, the protein sample must be enriched with ^{13}C and ^{15}N isotopes. Consequently, a bacterial expression system was developed to generate isotopically labeled protein samples. Using multidimensional NMR techniques, a complete structural analysis of the recombinantly expressed α_3W protein was performed [54]. The derived structural model verified that α_3W has a three-helix bundle topology and that the Trp-32 is located in the protein core. Interestingly, the NMR work also revealed that Trp-32 is involved in a π-cation interaction with a nearby lysine residue. The effects of the π-cation interaction on the reduction potential of Trp-32 were investigated by electrochemical and quantum mechanical methods. The calculations predict an increase on the reduction potential of Trp-32 when engaged in a π-cation interaction [54,55], which is consistent with the electrochemical characterization of α_3W [29]. π-charge interactions between aromatic and cationic residues are common in natural proteins [56,57] and may represent one mechanism by which redox proteins control the thermodynamic properties of their aromatic residues.

A 117-residue four-helix bundle, α_4W, has recently been constructed to complement the work on the three-helix-bundles and expand the family of model radical proteins. The molecular mass of α_4W is 13.0 kDa, which is almost twice the size of the 7.5-kDa α_3W protein. Due to the size of α_4W, a chemical synthesis is not expected to generate sufficient material for the initial structural evaluation of the design. To generate protein samples biochemically, the α_4W gene was assembled from six separate oligonucleotides using nested PCR techniques, cloned into an expression vector and transformed into *E. coli*. A preliminary structural characterization of the expressed and purified α_4W protein is indicative of a monomeric, well-structured α-helical protein (H.K. Privett, J. Järvet, B.R. Gibney and C. Tommos, unpublished data). A more detailed structural evaluation of a ^{13}C and ^{15}N double-labeled α_4W sample is currently in progress. In summary, the combination of de novo protein design with detailed structural, electrochemical and quantum mechanical analyses provides a novel method to probe amino-acid redox chemistry in proteins.

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